The ultrastructural evidence of progressive deterioration in the blastodermal cells of chicken eggs during pre-incubation storage.

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When chicken and turkey fertile eggs are held for extended periods of time under conditions commonly regarded as 'optimal', they are known to lose progressively their hatching potential. The latter eventually drops to zero (e.g. Funk and Forward, 1960). To the naked eye or under a low magnification with the light microscope, the gross morphology of blastoderms in the affected eggs reveals a growing deviation from the norm, particularly in the area opaca (Arora and Kosin, 1966). The spatial relationship among major blastodermal components is also affected (Weisbroth and Kosin, 1966). The cells of such blastoderms, seen under the light microscope, exhibit a wide range of "abnormalities", the frequency of which is directly related to the length of storage (Arora and Kosin, 1967). Cell necrosis becomes increasingly prevalent, with the chromatin showing changes which have been interpreted as degenerative (Konishi and Kosin, 1974). More recently, the ongoing analysis at this laboratory of the blastodermal material from chicken eggs subjected to pre-incubation storage has been based on electron microscopy. The present paper reports on this aspect of the study.

Materials and Methods

The eggs came from several chicken flocks, both mated and not mated at the University Poultry Plant. While most of the birds

involved were White Leghorns in their first year of production, some were New Hampshires and cross-breds of comparable age. Because no differences could be detected among the three types of eggs with respect to the criteria relevant to this study, the final data were pooled for the purpose of the present report.

In general, the eggs were collected within 2-3 hours of oviposition. The few specimens of blastodises (from non-mated birds) were obtained by processing the eggs immediately after their laying. Eggs from the mated pens intended for immediate fixation were delivered to the laboratory from the Plant in the afternoon of the day of laying, while those eggs to be stored were left in an egg holding room maintained at about 12.5°C. For most eggs that were subjected to storage the holding period did not exceed 21 days, although in a few individual tests it extended for as long as 70 days. Some of the stored eggs were subjected to a daily regimen of 60-minute warming at 39.5°C, after which the eggs were immediately returned for further storage. Such periodic warming was introduced into the over-all experimental design because an earlier study with turkey eggs showed that the warming treatment of eggs during storage helped to counteract to some extent the detrimental effect of prolonged storage on the developmental potential of their blastoderms (Kosin and St. Pierre, 1956). In addition to the above, still other eggs were placed for different periods of time (5 hours and up) in an incubator at 39.5°C after which they were either put through the fixation procedure or returned to storage.

In the laboratory, the procedure used during the pre-fixation and fixation phases underwent several changes since the start of the

study in 1972. At that time, aside from the work of Bellairs (1961), no reports existed in literature that dealt specifically with the preparation for the transmission electron microscopy of germ discs from non-mated birds as well as of unincubated and early incubated avian blastoderms. Hence a procedure had to be evolved that would be suitable for the objectives of the study.

In the case of eggs from mated pens, the fact whether or not the egg was fertile was established while the yolk (and the germ disc) was <u>in situ</u>. The classifying of the germ disc was done according to the method described by Kosin (1944), with the examination being carried out through an opening made in the shell at the large end of the egg.

Prior to 1975, the fixative of choice was an 8% solution of glutaraldehyde in 0.1M collidine buffer. Although several other concentrations of glutaraldehyde had been tried, ranging between 2% and 6%, the combination of the 8% fixative with the collidine buffer (the latter in preference to the phosphate buffer) gave the fastest penetration through the vitelline membrane.

Initially, the fixative ~~used to~~ was be injected under the blastoderm while the latter was <u>in situ</u> on the yolk within the shell. To facilitate this step it was found necessary to remove beforehand some of the overlying albumin, by pipetting. Unfortunately, this procedure proved to be too time consuming. Moreover, it sometimes led to an accidental rupturing of the vitelline membrane, with the consequent distortion of the blastoderm.

In another method that was tried, the albumin was flushed out of the shell by directing a gentle stream of tap water just under the floating yolk. The washing continued for 2-3 minutes, long enough

to replace all or most of the albumin with water; the latter was then pipetted off. One variant of the method called for adding enough of the fixative into the shell to cover the blastoderm. In the second variant, after the albumin had been washed out, the yolk would be first transferred to a watch glass - to remove the last traces of the thick albumin, and then into a preparation bowl containing the glutaraldehyde solution. Normally, four or five yolks could be accommodated in a 300 ml. bowl. This last mentioned procedure sometimes led to problems with stored or incubated eggs: the fragility of their vitelline membrane frequently resulted in its rupture and consequent loss of the specimen. In both variants, the blastoderm after a 15-30 minute fixation was cut out, lifted with a small spatula-sieve, and transferred into a vial containing a 0.1M phosphate buffer solution kept at 4°-5°C. The blastoderm remained within the solution for some 36 hours and then processed further.

However, because of the large amount of the fixative required by both of these procedures, they were eventually replaced by the one described below.

In this procedure, which is particularly suitable either for the newly laid eggs or those not subjected to incubation, the egg is opened, as usual, at the large end (this can be done with a special tool designed for lifting off a shell cap) and the yolk allowed to slip into a shallow, square Petri dish. Thus, the bulk of the albumin remains in the shell. If the blastoderm is not on the top while the yolk is resting in the dish, a sheet of parafilm is slid under the yolk to facilitate manipulating it, to bring the germ disc into the desired topmost position . At this stage, the

yolk is still surrounded by a layer of dense albumin. To remove this albumin a tip of the round nose scissors is inserted under it and just over the vitelline membrane. A cut of about 30-40 mm. is made in the ablumin, which then can be slipped off the yolk, exposing the blastoderm. A ring, 4 mm. in height, cut from the top of a standard plastic BEEM embedding capsule is now placed over the blastoderm - to serve as the reservoir ('well') for the fixative, which is immediately poured in.

Fixation is carried out with a 5% glutaraldehyde (in a 0.1M phosphate buffer, at pH 7.4), at 18°-20°C. The fixative remains in the well for 5-15 minutes, after which it is pipetted off and replaced by osmium tetroxide (1gm. OsO_4 in 1.5ml. CCl_4). To keep the osmium vapor within the well, the latter is covered by a cap, cut from a BEEM capsule. After 1-4 minutes, osmium tetroxide is replaced by a 30% ethanol.

Further dehydration is in an ethanol series of 50, 70 and 95%, 10 minutes in each. This is followed by two changes of absolute alcohol, 5 minutes each. The latter is then replaced by two changes, 10 minutes each, of propylene oxide. Finally, the tissue is infiltrated with an epon - propylene oxide mixture (1:1). The propylene oxide is allowed overnight to evaporate under a hood. Definitive embedding is in epon which is added into the well to embed the tissue. The plastic ring can now be removed, by cutting it from the blastoderm specimen. Until then the ring has to remain attached to the vitelline membrane, to prevent it from folding or curling during the dehydration.

Experience has shown that the entire procedure is best carried out at room temperature: when blastoderms were fixed or dehydrated at 4°-5°C, as had been done originally, it was observed that the cells lost a good deal of their stuctural detail. This was particularly true for the microtubules, which were at best rarely seen, and for the mitochondriae - their christae were less distinct following fixation etc. at low temperatures. The rise of a 5% osmium tetroxide

Fig. 7 Unincubated eggs stored 6 weeks. A) breakdown in mitochondria. The Golgi complex still recognizable (center). Singlet ribosomes. The endoplasmic reticulum almost completely lacks ribosomes. (13,000 x 2.5); C) chromosome-nucleolar complex. Note "cysts" on the outer nuclear membrane. (52,000 x 2.5); D) mitochondria with cristae and with "foamy" matrix. Singlet ribosomes. (13,000 x 2.5)

Fig. 8 Egg stored 12 weeks. A) Nucleolus and two "inclusions". (13,000 x 2.6). B) Yolk globules in the cytoplasm. (4500 x 2.5).

Fig. 9 Eggs first stored 23 days, then incubated 24 hours. A) the mitochondrial matrix breaks down. Numerous polysomes. (13,000 x 2.5); B) ribosomes along the endoplasmic reticulum. (9,700 x 2.5); C) numerous polysomes. Few ribosomes on the endoplasmic reticulum. (13,000 x 2.5).

Fig. 10 First, the eggs were stored 9 days, during which period they were subjected to the one-hour-per-day warming treatment. After that period they received additional 6 days of storage, followed by 24 hours of incubation. Note "normal" appearing mitochondria and Golgi apparatus and few polysomes. Evidence of ribosomes along the endoplasmic reticulum. (13,000 x 2.5).

Fig. 11 Eggs stored 23 days during which period they were subjected to one hour per day of warming. A) "bleb" on the outer nuclear membrane. (23,000 x 2.5); B) chromosomal complex. Ribosomes along the endoplasmic reticulum. (7,000 x 2.5); C) mitochondria with and without cristae. Ribosomes along the endoplasmic reticulum. Polysomes and singlet ribosomes. (7,000 x 2.5).

Fig. 1 Freshly laid, unincubated fertile egg. A) a typical cell (5900x) Note the large nucleolus, the Golgi complex (lower right corner) and several mitochondria. The ribosomes are widely distributed. The presence of vacuoles is characteristic of such cells (5900x); B) desmosome (34.000x); C) a mitochondrion near the nuclear envelope with three nuclear pores showing.

Fig. 2 A typical cell in a freshly laid, unincubated infertile egg. A) mitochondria, which lack cristae; B) microtubule; C) mitochondrion and the developing endoplasmic reticulum. Note the singlet ribosomes in all three pictures. Magnification: 13,000 x 2.5.

Fig. 3 Freshly laid egg, incubated 24 hours. Ribosomes above the endoplasmic reticulum. Nuclear pores and annular material. Widened perinuclear space. Microtubules. (19,000 x 2.6).

Fig. 4 Egg stored 48 hours and then incubated 38 hours. Note the close association of ribosomes along the nuclear envelope (left) and along the endoplasmic reticulum. (19.000 x 2.6).

Fig. 5 Egg stored 48 hours. A) mitochondria lack cristae. Few polysomes (13,000 x 2.5); B) a "cyst" forming on the outer of nuclear membrane. Magnification: 19,000 x 2.6.

Figs 11–14 stored 16 days. Unincubated eggs. A) Numerous short microtubules. (19,000 x 2.6); 12 B) "convoluted" cristae. (19,000 x 2.6); C) nuclear pores and electron dense material opposite them on the cytoplasmal side. (19,000 x 2.6); 13 D) two nucleoli with condensed chromosomes. A "hole" in the nucleoplasm. (4,500 x 2.5) A,B, C: eggs stored 16 days.

Fig 15 Figs 15, 16 eggs stored 3 weeks. E) nucleolus and remnants of condensed chromosomes (13,000 x 2.6). ~~Egg stored 3 weeks.~~ F) a lobed nucleus, containing a chromosomal-nucleolar complex. Note an "inclusion" in the top portion of the nucleus. (7,000 x 2.5). Egg stored 2 weeks.

Fig. 12 First, the eggs were stored 8 days, during which period they were subjected to one hour per day of warming. Then the eggs were stored for 14 days. A and C) "outpouching" of

the nuclear membrane. Note the presence of mitochondria (?) close by;

B) a remnant of

the nucleolus (?) attached to the inner nuclear membrane; D) an inclusion in the nucleus. Magnification: 19,000 x 2.5.

in a 0.1M phosphate buffer used in some of the initial tests resulted in the definition of various membranous structures that was less satisfactory than that obtained with the higher concentrations of OsO_4 (e.g. 40%-80% in the more recent tests).

Results

Cells from blastoderms of the unincubated, non-stored eggs served as a standard of comparison for cells of the treated eggs, i.e. Those (1) stored at 12.5°C with or without periodic warming or (2) subjected to a single, short-term incubation at 39.5°C.

A typical cell from the blastoderm of a freshly laid (i.e. egg fixed 2-3 hours after oviposition) and unincubated egg is seen in Fig. 1. Its nucleus is regular in shape, often ovoid, and may show one or more chromosomal complexes which are in close association with the nucleoli. The latter are mostly granular in type, with occasional admixture of fibrilar areas.

The inner nuclear membrane is, as a rule, free of the chromatin material. The nuclear pores are numerous and prominent. The few ribosomes that can be seen are, to a large extent, randomly distributed, although occasionally they aggregate near the large vacuoles which are common in the cytoplasm of most blastodermal cells. Besides these vacuoles, the cytoplasm also has several types of yolk globules, the most common of which have electron dense lipoid cores. The polysomes are still scarce. The mitochondria tend to accumulate near the nucleus and the Golgi apparatus. The average mitochondrion has the well developed outer and innner membranes, the latter giving rise to one or more cristae. These exhibit limited spatial development, with only an occasional one traversing the width of

a mitochondrion. The apparent homogeneity of the mitochondrial matrix is often disrupted by irregular, electron translucent globular ('foamy') formations. In such cases the cristae are completely absent. The microtubules may be present, especially near the nuclear envelope. ~~Both the smooth and rough endoplasmic reticulum (ER) are recognizable, of which the former is much more ubiquitous. The rough ER appears mostly near the nucleus and near the aggregations of mitochondria.~~ An occasional desmosomal junction has been observed.

Along with the specimens of the type described above, a limited number of germ discs from non-mated birds has been studied. ~~Except for some not unexpected differences,~~ In some respects, the blastodiscoidal cells duplicate the morphology of their blastodermal counter-parts. The nucleus was not located in the specimens obtained from these blastodiscs which, under the circumstances, was not surprising: The chances of coming across the nucleus of the sole blastodiscoidal (maternal) cell would be small, especially in preparations that were not cut serially, as was the case in the present instance. The mitochondria were small, roundish, lacking ~~any~~ signs of cristae (Fig. 2). The ribosomes, all singlets, were numerous and widely distributed. The endoplasmic reticulum was almost entirely of the smooth type. When present, the microtubules were long and well defined. The Golgi complex was not observed.

Even a brief, 5-20 hour incubation at 39.5°C brought about dramatic changes to the blastodermal cell. The formation of polysomes was stepped up, with most of these now appearing as clusters of 5 or more ribosomes. Single ribosomes, which still greatly out numbered polysomes, were often seen to be closely associated with the endoplasmic reticulum [redacted] (Fig 3). The latter (ER) was extensive in its distribution and ramification. The outer nuclear membrane was more clearly delineated, primarily because of the widening of the peri-nuclear space. The numerous nuclear pores contained well defined annular

material. One often encountered accumulations of electron dense matter opposite these pores on both sides of the nuclear envelope.

A thin band of the chromatin material usually lined the inner nuclear membrane. The nucleoli were prominent, presenting a meshwork appearance, made up of fibrilar and granular components. The microtubules were pervasive and widely distributed, especially near the nucleus. The infoldings of the inner mitochondrial membrane have grown into the cristae, many of which by now have developed complex configurations. At least some of the cristae have come to traverse the entire width of the mitochondrion. The Golgi complex(es) appeared as extensive network of cisternae, which varied both in configuration and size. These organelles usually were found in close association with the mitochondria.

Storing eggs first at 12.5°C for 24-48 hours and then incubating them at 39.5°C for 20-38 hours produced blastoderms in which cells were closely comparable to those obtained in the freshly laid, i.e. non-stored eggs subjected to 24 hours of incubation (Fig. 4). What was particularly striking in the case of all these <u>incubated</u> eggs was the extensive distribution of single ribosomes along both the [redacted] ER and, to a lesser degree, the outer nuclear membrane. Those eggs stored for 24-48 hours <u>without</u> incubation duplicated the already described pattern found in eggs fixed immediately after oviposition. At the same time, a short term storage, of up to 48 hours, did not prevent polysome formation (Fig. 5). In fact in such eggs, this process appeared to be more advanced than in the blastodermal cells of freshly laid non-incubated eggs (cf. Fig. 1). On the other hand there was little evidence of the ribosomal association with the [redacted] ER.

The cells of eggs which were incubated for 24 hours immediately after oviposition and then fixed (in other words, these were completely non-stored eggs), did not differ in their morphology from the cells of

eggs which were first subjected to a 7-10 day storage and then incubated for 24 hours. However, longer storage with or without incubation produced cellular changes, the extent of which was directly related to the length of storage.

In the unincubated eggs stored beyond 10 days, the occurence of nuclei with amorphous electron dense inclusious become frequent (Fig. 6). Moreover, a higher proportion of the nuclei were seen to develop ill-defined lobes and other distortions of form. The nucleoli were now more prominent than ever before, their assoication with the chromosomal complexles remaining close. The chromosomes were more condensed and, hence, electron dense. The perinuclear chromatin materal in the nuclei was scarce. The nuclear pores were large and they often had electron dense material juxtaposed opposite them on the cytoplasm side of the nuclear membrane. The ribosomes, predominantly singlets, were randomly distributed, without showing any evidence of aggregating near or along the [redacted] ER. Many of the mitochondria lacked both the cristae and the matrix. When cristae were presnt, they tended to be short and convoluted to a degree never observed in the cells of eggs fixed shortly after the oviposition. When visible, the microtubules appeared short, lacking easily definable orientation. The Golgi apparatus was still extant.

Although this general picture of intra-cellular breakdown gained momentum with continued storage, the basic organizational design of these cells was still recognizable as late as six weeks after the beginning of post-oviposital storage (Fig. 7). This applied even to the intra-structral integrity of mitochondria and of the Golgi complex. Although the nucleoli remained prominent, the individual chromosomes were no longer identifiable. By the eighth week of storage, the nuclear envelope began to show signs

of a breakdown. However, in the individual cells the "normal appearance" of that structure, including the nuclear pores, was retained up to twelve weeks of storage time (Fig. 8). In all of the aging cells of eggs stored for periods shorter than twelve weeks, the nuclei usually contained one or more chromosomal-nuclar complex which became increasingly more dense in appearance with the age of the egg. The mitochondria, ribosomes and other cytoplasmic organelles were recognizable in eggs stored beyond eight weeks. The cytoplasm in such eggs was gradually filled with yolk globules and large vacuoles. In the cells of eggs retained under storage conditions for twelve weeks, the nuclear matrix showed no semblance of organization.

Storing eggs beyond 10 days, e.g. for 14 or 23 days, did not destroy the capacity of their cells to respond to a 24-hour-long incubation. In fact, following incubation such cells resembled in appearance much 'younger' cells, i.e. those found in eggs either fixed immediately after oviposition or incubated for 24 hours following a storage of less than ten days' duration. What differences were detected, were limited largely to mitochondria, many of which in the older eggs, those kept in storage beyond ten days, were seen to be deficient both in the cristae and in the matrix (Fig. 9). The close association of singlet ribosomes with the ([redacted]) ER, which characterizes the cytotoplasm of cells in vigorously developing blastoderms, now was much less extensive. However, cells in the eggs stored for 28 days never exhibited any morphological signs of response to incubation.

The regimen of daily one hour warming at 39.5°C for up to 23 days, with the remaining 23 hours of the 24-hour period at 12.5°C, resulted in a pictrue that revealed evidence of intra-cellular activity of a type normally associated with blastodermal development, including chromosomal involvement

(Fig. 10). The mitochondria, on the other hand, began to show signs of internal deterioration much sooner.

When these eggs were subjected at the end of their 23-day-long storage to 24 hours of now continuing incubation, the cells exhibited the presence of numerous, widely distributed polysomes and occasional intact mitochondria (Fig. 11). But the association of singlet-ribosomes with the [redacted] ER was minimal.

The results of a small scale hatching test supported the above observations on the ameliorative effect of daily warming on maintaining the developmental capacity of cells following storage. The test, which consisted of 14-day storage followed by the regular period of 21-day incubation, involved 40 fertile eggs divided evenly between two treatment groups: group 1 - the eggs were on the daily regimen of one hour warming (at 39.5°C) followed by 23 hours of storage (at 12.5°C): group 2 - the eggs (controls) were stored at 12.5°C for the duration of the test.

By the end of the first week of incubation, 12 eggs in group 1 contained dead embryos; the remaining 8 eggs produced healthy chicks. By contrast, in group 2 at the end of the first week of incubation there were 10 dead embryos, two more were dead by day 14 and 4 dead by day 20. Two fully formed chicks were 'dead-in-shell' on day 21; two chicks hatched.

The signs of activity, induced in the blastodermal cells of stored eggs by the daily warming routine, were observed, at times, for as long as 14 days after the termination of that treatment during which time (i.e. 14 days) the eggs remained constantly exposed to 12.5°C [redacted]. These signs included the presence (1) of polysomes (although their frequency was low), (2) of [redacted] mitochondria (That lacked cristae, (3) of greatly enlarged chromosome-nucleolar

complexes. However one feature that is normally associated with cell morphology of actively developing blastoderms - the lining of the [redacted] ER by singlet-ribosomes - was absent.

A puzzling side issue was encountered in the above series of specimens: the outpouching of the nucleus into the cytoplasm. These formations possessed a double membrane which was obviously derived from the nuclear envelope (Fig. 12). Usually, this process was seen to occur in areas well endowed with mitochondria. In at least two specimens, mitochondria - like inclusions were spotted within the nucleus, closely adjoining the nuclear envelope. And again, several mitochondria were present near by, on the cytoplasm side of the outer nuclear membrane. In one of these specimens an electron dense mass appeared to be passing through the nuclear envelope.

To achieve the ameliorative effect of periodic warming on the cells of stored eggs (i.e. exposing eggs for one hour per day to 39.5° C for the duration of storage), the treatment had to be initiated not later than 48 hours after the oviposition. Within that time frame, the cells exhibited all of the characteristics of normal intracellular activity, including the presence of numerous polysomes and evidence of extensive association of singlet-ribosomes with the [redacted] ER [redacted]. By contrast, delaying the start of the warming regimen beyond the 48-hour span of time, e.g. for 6-8 days, resulted in intra-cellular 'dormancy' that differed little from that seen in the cells of non-incubated eggs subjected to extended storage (cf. Fig. 6).

Discussion

We propose the term induced senescence to describe the condition of continuous deterioration observed in the blastodermal cells of avian eggs subjected to pre-incubation storage. It is not synonymous with that of immature cellular aging, as used by Gelfant and Smith (1972), which applies to a process occurring during normal embryogenesis. Induced senescence designates the complex of morphological changes in the cells of blastoderms in which the course of normal development has been prevented, for longer or shorter periods of time, by sub-optimal temperature.

In the environment which best preserves hatching potential, i. e. the ambient temperature of 12°- 13°C and the relative humidity of 80%, these cells become non-cycling, incapable of undergoing mitosis (for terminology, see Gelfant and Smith, loc. cit., and Bowman et al., 1976). As the duration of storage increases, this process grows in intensity until, after some 30 days, the blastoderms will no longer re-initiate active embryogenesis upon being exposed in-situ to 'normal' incubation temperature (39.5°C). To all intents and purposes, such blastoderms are dead.

The fact that this progressive incapacitation of the embryo's organism can be slowed by the regimen of periodic warming - one hour per day, at 39.5°C - suggests that it is a well integrated phenomenon, the effects of which are cumulative. The capacity of cells to respond to the stimulus of the incubation level temperature in itself does not guarantee subsequent normal embryogenesis. It is true that the ultrastructural evidence obtained in the course of the present study clearly shows that such cells again become cycling, something that should auger well for the future of the embryo of which they are a part. And yet, reports in the literature (e.g. Kosin and St. Pierre, 1956; Milby and Sherwood, 1960; Vladimirova, 1961; Kan et al.,

1962) as well as the limited data collected in this study, demonstrate that warming eggs does not completely counteract the negative effects of a prolonged storage on the viability of the embryo.

At the ultrastructural level, the morphological changes detected in the cells of the affected blastoderms were seen to be largely centered in the cytoplasm. This contradicts the conclusions of earlier studies based on light microscopy (e.g. Glucksman, 1951; Arora and Kosin, 1967). In the present work, a series of major changes were observed to take place in the internal structure of mitochondria and in the organization and distribution of ribosomes in eggs kept in pre-incubation storage beyond the first 4-5 post-ovipositai days. The chromosomal complex, on the other hand, remained relatively constant in appearance, at least during the initial stage of such storage.

It is possible that the referred to disagreement on the intra-cellular locale of significant changes may be due to the inherent limitations of light microscopy: the criteria found useful in the analysis based on electron microscopy would be beyond the power of resolution of the light microscope. The latter would be able to pick out such signs of growing cellular moribundity as chromosome clumping and distortion in the shape of the nucleus (Konishi and Kosin, 1974). However, the more discrete and dimensionally smaller criteria of change would remain undetected. All this does not rule out the possibility and the probability that nucleus is involved in the over-all process of induced senescence. However, the morphological approach utilized in the present study has not revealed any major signs of such involvement, except for the condensation of the chromosome-nucleolar complex in the eggs exposed to extended periods of storage.

In this connection, one is cognizant of the fact that Wright and Hayflick (1975) have concluded that during the growing senescence of WI - 38 fibroblasts

(from human fetal lung), the nucleus was the primary seat of the observed changes. To what extent (1) the 'natural' vs. the 'induced' senescence, (2) the specie and cell type differences, and (3) the differences in the procedures used caused this apparent contradiction in the 'aging process' can be answered only by future studies. The situation is rendered still more complex, when Chen and Ruddle (1974), also working with the WI - 38 cells, concluded that structurally at least the chromosomes do not reflect that process.

All other observed changes in the 'senescing' cells, such as pertained to the distribution of the non-chromosomal chromatin, as well as changes in the distribution of cytoplasmic electron-dense aggregates vis-a-vis nuclear pores, and in the shape of the nucleus, appeared to be reflecting the over-all condition of the affected cell.

In our study, one of the observable cytoplasmic organelles was the ribosome. Only singlet ribosomes were encountered in the maternal cell of eggs laid by non-mated hens. Some polysomes appeared in the blastodermal cells of freshly laid but non-incubated eggs. The formation of such ribosomal clusters at the expense of monosomes was stimulated after the egg became exposed, for as short a period as one hour, to the normal incubation temperature.

The fact that polysomes could be identified in non-incubated fertile eggs should not be surprizing: the internal temperature of the egg at oviposition is about 40°C, the level that was found in the present work to be conducive to the formation of polysomes. One can speculate that as the egg cools, after oviposition, this process gradually ceases, to be resumed upon incubation.

It will be recalled that evidence was presented on the growing association in the incubating egg between the endoplasmic reticulum and the ribosomes: the latter were seen to line up, in increasing numbers, the membranous organelle. This occurred in the cells of eggs kept at 39.5°C. Bell _et al._ (1965) observed

that polysomes were insensitive to ribonuclease: they reported that these organoids were 'inactive' so long as they remained in such aggregates. After the ribosomal clusters opened up, the ribosomes arranged themselves into extended, chain-like structures and became functional, i.e. capable of synthesizing protein. The more recent evidence on the rearrangement of polysomes along the endoplasmic reticulum, presented by Birge and Doolin (1974), seems relevant here. They suggest that the establishment of such close relationship between those two cytoplasmic components indicates a synthesis of "intra-cellular products associated with proliferation and/or cellular differentiation." This interpretation, if correct, would help to explain the relevance of the ribosomal changes, described above, to the procedure recommended for handling hatching eggs in storage: keeping eggs at 12°- 13°C would minimize the danger of initiating the conversion of clustered (i.e. 'inactive') polysomes into 'active' ribosomes (those strung out along the endoplasmic reticulum), an essential step for re-activating embryogenesis. In the absence of the normal incubation temperature, such embryonic development would not be advantageous for the blastoderm: the immediate needs of the blastoderm cells within the stored hatching egg would be served best by retaining their ribosomes either as singlets or as ribosomal clusters. Keeping stored hatching eggs at 12o-13°C achieves this. A reasonable explanation for the continuing existance of such synchronization directed, first, at preserving embryo's viability and, second, at facilitating normal embryogenesis, once the process has started, is that it could only have evolved through natural selection.

The time scale of changes in the mitochondrion parallels that for the ribosomes. In the infertile egg, the mitochondria lack detectable cristae. These first appear in the newly laid fertile eggs in the 'incomplete' form,

i.e. without traversing the width of the organoid. The traversal by these septate cristae is completed only after the egg has been exposed to 39.5°C.

The mitochondrion is known to be involved in the oxidative metabolism of the cell; the presence of completed and intact cristae is essential for that function (for the literature on this see Borst, 1969). It is surmized, therefore, that the observed parallelism of the events affecting the ribosomes and the mitochondria is not coincidental but, rather, it reflects basic functional changes in the blastodermal cell.

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Summary

The principal objective was to study the ultrastructural morphology of blastodermal cells in chicken eggs subjected to pre-incubation storage at 12.5°C. The storage period ranged between 5 hours and 12 weeks.

Some of these eggs received, in addition, a 'warming' treatment: exposure for one hour each day to 39.5°C (normal incubation temperature). All tests had appropriate controls.

The blastodermal specimens were fixed in a 5% glutaraldehyde and post-fixed in 60-80% osmium tetroxide. Dehydration was in ethanol, followed by infiltration, first with propylene oxide and then with a 1:1 mixture of epon-propylene oxide. Definitive embedding was in epon. The entire procedure was at room temperature.

Cells in the blastoderms of freshly laid non-incubated eggs had numerous singlet ribosomes but few polysomes. Their mitochondria lacked completed cristae. Incubating such eggs for 24- 48 hours, as well as those first subjected to 5-24 hours of storage, enhanced the formation of, in progressively increasing numbers, polysomes and led to the lining up of the endoplasmic reticulum by ribosomes. This effect was directly related to the length of incubation.

In the <u>blastodiscoidal</u> cells of non-incubated eggs, there were singlet ribosomes but no polysomes and their mitochondria, which were small in size, lacked cristae completely.

Storing fertile eggs beyond 10 days led to deterioration within the nucleus and the cytoplasm: the chromosomes became more condensed and electron dense, the nuclear envelope developed distortions, the perinuclear chromatin disappeared, the ribosomes failed to line up the endoplasmic reticulum,

the mitochondria lost their cristae. All these changes were progressive in intensity, being directly related to the duration of storage.

This process of cellular retrogression was slowed down, to some extent, by the regimen of periodic daily warming of stored eggs. The ameliorative effect of that treatment was supported by the results obtained from a small scale hatching test conducted as a part of the over-all study.

The morphological approach utilized in this study for examining the ultrastructural changes in the course of induced senescence of blastoderms, brought about by storage, seems to point primarily to the cytoplasm as the initial locus of progressive cellular incapacitation.

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Abstract

Kosin, I. L. and J. D. Huber, (Department of Animal Sciences and Electron Microscope Center, Washington State University, Pullman, WA 99164, U.S.A.).
The ultrastructural evidence of progressive deterioration in the blastodermal cells of chicken eggs during pre-incubation storage.

The principal objective was to study (1) the ultrasturctural morphology of blastodermal cells in eggs subjected to pre-incubation storage at 12.5°C and (2) the possible ameliorative effect on such cells of a periodic warming (at 39.5°C) of the stored eggs. The storage brought about many clear cut changes both in the nuceus and the cytoplasm, particularly in the latter. The ribosomes and the mitochondria were especially affected. The warming regimen had a degree of ameliorative effect on these retrogressive changes. It was concluded that the <u>induced senescence</u> of blastodermal cells (caused by storage) was initially expressed in the cytoplasm.

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